-- Figure 1 shows the effect of temperature on the staining of CTL clone 003 by tetramers incorporating peptide variants. The cytotoxicity and tetramer staining of CTL clone 003 specific for the HLA-A2.1-restricted HIV epitope SLYNTVATL (SEQ ID NO:1) were assessed. A, Specific lysis of HLA-A2.1-matched targets pulsed with 10-fold dilutions of variant peptides at an E:T ratio of 3:1. Data points represent the mean of three replicates. B, Temperature dependence of staining with tetramers folded around SLYNTVATL (SEQ ID NO:1) variant peptides and the A2 HIV-1 reverse transcriptase peptide ILKEPVHGV (SEQ ID NO:2) (MFI) for SL*Y* and SL*H* variant tetramer staining at 4°C, 23°C, and 37°C.--

Please replace the paragraph beginning on page 18, line 2 with the following rewritten paragraph:

-- Figure 2 shows that effect of temperature on the staining of CTL clone 5D8 by tetramers incorporating peptide variants. The cytotoxicity and tetramer staining of CTL clone 5D8 specific for the HLA-A2.1-restricted HIV epitope SLYNTVATL (SEQ ID NO:1) were assessed. A, specific lysis of HLA-A2.1-matched targets pulsed with 10-fold dilutions of various peptides at an E:T ratio of 3:1. Data points represent the mean of three replicates. B, Temperature dependence of staining with tetramers folded around variant peptides. Staining is expressed as mean fluorescence for a homogenous cell population and is an average of two experiments.--

Please replace the paragraph beginning on page 18, line 11 with the following rewritten paragraph:

-- Figure 3 shows the effect of temperature on the staining of polyclonal CTL line 868 by tetramers incorporating peptide variants. The cytotoxicity and tetramer staining of polyclonal CTL clone 868 specific for the HLA-A2.1-restricted HIV epitope SLYNTVATL (SEQ ID NO:1) were assessed. A, Specific lysis of HLA-A2.1-matched targets pulsed with 10-fold dilutions of variant peptides at an E:T ratio of 3:1. Data points represent the mean of three replicates. B, Temperature dependence of staining with tetramers folded around variant peptides. Staining with tetramer containing an

HLA-A2.1-restricted epitope from HIV-1 reverse transcriptase (ILKEPVHGV) (SEQ ID NO:3) is shown for comparison. Staining is expressed as the percentage of $CD8^+$ lymphocytes visualized with tetramer for a heterogeneous cell population. C, Density plots fir SL*Y* and SL*H* variant tetramer staining at 4°C, 23°C, and 37°C.--

Please replace the paragraph beginning on page 18, line 23, with the following rewritten paragraph:

--Figure 4 shows time course of tetramer staining at 37°C. The kinetics of tetramer staining at 37oC were investigated by incubating CTL clone 003 with SLYNTVATL (SEQ ID NO:1) tetramer for various lengths of time (0.5, 5, 10 and 20 min). SLYNTVATL (SEQ ID NO:1) tetramer was not added to the "0 min" sample. The experiment was conducted in the presence of 0.1% azide throughout.--

Please replace the paragraph beginning on page 18, line 29 with the following rewritten paragraph:

--Figure 5 shows that tetramers incubated at 37°C are rapidly internalized by Ar-specific CTLS. A human CTL clone specific for the HLA-A2.1-restricted IMP epitope GILGFVFTL (SEQ ID NO:3) was incubated with FITC-labeled GILGFVFTL (SEQ ID NO:3) tetramer for 15 min at 37°C and subsequently fixed and subjected to confocal staining with and without Texas Red costaining for the transferrin receptor. The top four panels show that when excited with the 488-nm line, only the FITC signal is detected; likewise, when excited with the 568-nm line, only the Texas Ted signal is detected, confirming lack of "bleed through" between the channels. The bottom panels show excitation at 488 nm plus 568 nm, demonstrating overlap between the signals, indicating that endocytosed tetramer is reaching early endosomes that stain for transferrin receptor.--

Please replace the paragraph beginning on page 20, line 27 with the following rewritten paragraph:

-- Peptides were synthesized by standard fluorenylmethoxycarbonyl chemistry and subsequently used in chromium release assays and in synthesis of tetramers. The peptides used, referred to subsequently in the text by their first three letters only, included: SL*Y*NTVATL (SEQ ID NO:1), an HLA-A2.1-restricted epitope from the HIV p17 gag matrix protein, and its naturally occurring variants SL*H*NTVATL (SEQ ID NO:4), SL*S*NTVATAL (SEQ ID NO:5), SL*F*NTVATL (SEQ ID NO:6), SL*F*NAVATL (SEQ ID NO:7), and SLNYT*I*AVL (SEQ ID NO:8) (24, 26, 27); ILKEPVHGV (SEQ ID NO:2), an HLA-A2.1-restricted epitope from the HIV pol protein (2); GLCTLVAML (SEQ ID NO:9), an HLA-A2.1 epitope from the BMLF1 protein of EBV; and GILGFVFTL (SEQ ID NO:3), an HLA-A2.1-restricted epitope from the influenza matrix protein (IMP).--

Please replace the paragraph beginning on page 21, line 7, with the following rewritten paragraph:

-- Tetrameric peptide-MHC class I complexes were made as described previously. Briefly, recombinant β_2-microglobulin and the extracellular portion of the MHC class I heavy chain containing the BirA recognition sequence in frame at its C terminus were expressed in *Escherichia coli* as insoluble aggregates that formed inclusion bodies. Purified inclusion bodies were solubilized in urea, and monomeric HLA class I complexes were refolded around peptide by dilution of denaturing conditions. After buffer exchange, a specific lysine residue in the heavy chain C-terminal tag was biotinylated with BirA enzyme. Monomeric complexes were purified by gel filtration and anion exchange chromatography. Tetrameric arrays of biotinylated peptide-MHC class I complexes were formed by the addition of PE- or FITC-labeled avidin (extravidin, Sigma, St. Louis, MO). Tetramers are referred to by the first three letters of their peptide (e.g., GIL for the HLA-A2.1 GILGFVFTL (SEQ ID NO:3) tetramer).--

Please replace the paragraph beginning on page 22, line 18 with the following rewritten paragraph:

-- The majority of HLA-A*0201 HIV-1-infected patients mount a CTL response to an immunodominant epitope (SLYNTVATL) (SEQ ID NO:1) encoded in the p17 gag matrix protein. We have documented naturally occurring mutations within this epitope that can lead to escape from, or altered recognition by, patient CTLs. CTL clones 003 and 5D8 and a CTL line from patient 868 show different patterns of recognition of naturally occurring SLYNTVATL (SEQ ID NO:1) variants (Figs. 1-3), consistent with their different origins and TCRs. We used these CTL and HLA-A2 tetramers folded around wild-type and variant peptides to examine the specificity of direct CTL visualization in vitro.--

Please replace the paragraph beginning on page 22, line 27, with the following rewritten paragraph:

-- APLs, which act as weak agonist or antagonist ligands, have a lower affinity for the TCR, while they can bind to MHC with similar affinities. Tetramers of poorly recognized APLs van stain CTLs at 4°C (see SL*H* in Fig. 1, SL*S* in Fig. 2, and SL*F* in Fig. 3). Surprisingly, APL-MHC tetramers of ligands that do not stimulate CTL-mediated lysis, such as the SL*H* variant with 868 CTL, can also stain CTLs efficiently at 4°C (Fig. 3). We were unable to demonstrate antagonism of these CTLs with the SL*H* variant. None of these A2 gag CTLs were observed to stain with A2 tetramers of the unrelated epitopes ILKEPVHGV (SEQ ID NO:2) (Figs. 1-3), GILGFVFTL (SEQ ID NO:3), or GLCTLVAML (SEQ ID NO:9).--

Please replace the paragraph beginning on page 23, line 8, with the following rewritten paragraph:

-- The binding of TCRs to their peptide-MHC ligands at 25°C is characterized by low affinity, slow kinetics, and a high degree of cross-reactivity. The dramatic increase in binding minetics with temperature prompted us to investigate the effect of temperature on the ability of peptide-MHC tetramers to from stable complexes with cell surface TCR. At 4°C, both CTL clones stained with tetramers containing SLYNTVATL (SEQ ID NO:1) variant peptides that they recognised only weakly in chromium release (see variant

SL*H* in Fig. 1 and SL*S* in Fig. 2). For both clones, increasing the incubation temperature to 25oC and subsequently to 37°C reduced the staining by tetramers incorporating weakly recognised peptide

Please replace the paragraph beginning on page 23, line 25, with the following rewritten paragraph:

-- The potential effect of this phenomenon on the staining of biological samples is seen with a polyclonal CTL line from patient 868 that also recognises SLYNTVATL (SEQ ID NO:1) peptide variants to different degrees (Fig. 3). This polyclonal line contains ~31% of SLYNTVATL (SEQ ID NO:1)-specific CTLs, as seen by staining with the SL*Y* tetramer (Fig. 3B). When incubated at 4°C, tetramers made from the SLH variant peptide, which does not stimulate CTL-mediated lysis, stain a population of similar size (Fig. 3B). Increasing the temperature of the tetramer incubation reduces the proportion of this line that stains with the SL*H* tetramer, so that at 37°C, there is no staining with this unrecognized peptide variants (Fig. 3, B-C). The results for the three different TCRs and several APLs in Fig. 1-3 show that improvements in staining specificity for strongly recognised ligands with increasing temperature are neither TCR- nor APL-dependent.--

Please replace the paragraph beginning on page 24, line 10, with the following rewritten paragraph:

-- It is important to note that all SLYNTVATL (SEQ ID NO:1) variant tetramers used are sufficiently stable at 37°C to stain appropriate CTLs brightly (Fig. 1-2). Hence where increasing temperature reduces tetramer staining, in the case of variant peptides that are poorly recognised by the CTLs, this is not due to tetramer instability at higher temperature (e.g., the SLF tetramer stains 003 CTLs brightly at 37°C but does not stain 868 CTLs at this temperature). Preincubation of tetramers at 37°C for 60 min before staining CTLs also had no demonstrable effects on subsequent staining (data not shown), confirming the stability of the reagents under the experimental conditions employed.--

Please replace the paragraph beginning at page 24, line 28, with the following rewritten paragraph:

-- The rapid bright stable staining observed of CTLS at 37°C led us to examine whether tetramers were being internalized after interacting with the TCR. An IMP-specific CTL clone that stained in the FACS analysis with GILGFVFTL (SEQ ID NO:3) tetramer but not with other HLA-A2 tetramers was incubated with this tetramer for 15 min at 37°C and examined by confocal microscopy, with and without double staining, to identify intracellular compartments. Tetramer was indeed internalised by this clone within 15 min at 37°C, and was visible in intracellular vesicles (Fig. 5). These vesicles overlapped in distribution with vesicles containing the transferrin receptor, although not all of these early endosomes were labeled with tetramer. Internalized tetramer did not colocalize to secretory lysosomes containing perforin or granzyme B. Incubation with tetramer for 15 min at 4°C did not induce detectable internalization of tetramer.--

Please replace the paragraph beginning on page 46, line 21, with the following rewritten paragraph:

-- Using the polymerase chain reaction, DNA coding for the 15 residue BirA substrate peptide (BSP) (SEQ ID NO:10) LHHILDAQKMVWNHR, was fused (Scartz, 1993) to the 3' end of a previously described gene for the expression of soluble I-E^{κ}_{α} in *E. coli* (Altman, 1993). An antisense oligonucleotide was designed (SEQ ID NO:11) 5' CCGGAATTCTTAACGATGATTCCACACCATTTTCTGTGCATCCAGAATATGAT GCAGGAGGAGGGTTTTCTCTTC 3'. In the sense direction the oligonucleotide provides for the 18 bases corresponding to the C-terminus of soluble Ec-I-E^{κ}_{α}, 45 bases encoding the BSP, a stop codon and an EcoRI restriction site, plus flanking bases. The sense primer for the PCR, (SEQ ID NO:12) CATATGGCTAGCATCAAAGAGGAACACACCAT has been previously described (Altman, 1993).--

Please replace the paragraph beginning on page 49, line 10, with the following rewritten paragraph:

-- Enzymatically biotinylated proteins are loaded with the 88-103 peptide from moth cytochrome C, ANERADLIAYLKQATK (SEQ ID NO:13) according to established protocols (Wettstein [1991], *supra*; Reay [1992] supra). The empty αβ heterodimer was incubated with McIlvaine's citric acid-phosphate buffer (CPB) at 37°C in presilanized microfuge tubes. Reactions were adjected to pH 7 by addition of 2 M Na_2HPO_4. Peptide loaded bio-*Ec*-I-E^k-BSP molecules are purified by gel filtration on Superdex 200 columns, in a PBS mobile phase.--